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-1-

COMPOUNDS THAT MODULATE ENDOTHELIN ACTIVITY

This application is a continuation-in-part of United States Application Serial No. 07/886,387, filed May 19, 1992, by Ming Fai Chan and Vitukudi N. Balagi, entitled "COMPOUNDS THAT MODULATE ENDOTHELIN ACTIVITY".

FIELD OF THE INVENTION

The present invention relates to compounds that modulate or alter the activity of the endothelin family of peptides. More particularly, compounds that inhibit the activity of endothelin and that thereby possess therapeutic utility are provided.

5 BACKGROUND OF THE INVENTION

The vascular endothelium releases a variety of vasoactive substances, including the endothelium-derived vasoconstrictor peptide, endothelin (ET) (see, e.g., Vanhoutte et al. (1986) Annual Rev. Physiol. 48: 307-320; Furchgott and Zawadski (1980) Nature 288: 373-376).

- 10 Endothelin-1, which is a potent twenty-one amino acid peptide vasoconstrictor that was originally identified in the culture supernatant of porcine aortic endothelial cells (see, Yanagisawa et al. (1988) Nature 332: 411-415), is the most potent vasopressor known. It is produced by numerous cell types, including the cells of the endothelium, trachea,
- 15 kidney and brain. Endothelin is synthesized as a precursor of 203 amino acids, called preproendothelin, containing a signal sequence which is cleaved by an endogenous protease to produce a 38 (human) or 39 (porcine) amino acid peptide. This intermediate, referred to as big endothelin, is processed to the mature biologically active form in vivo by
- 20 a putative endothelin-converting enzyme (ECE; see, e.g., Kashiwabara et al. (1989) FEBS Ltrs. 247: 337-340), which appears to be a metal-dependent neutral protease. Processing is required for induction of physiological responses (see, e.g., von Geldern et al. (1991) Peptide Res. 4: 32-35). In porcine aortic endothelial cells, the 39 amino acid

-2-

intermediate, big endothelin, is hydrolyzed at the Trp²¹-Val²² bond to generate endothelin-1 and a C-terminal fragment. A similar cleavage occurs in human cells from a 38 amino acid intermediate.

Three distinct endothelin isopeptides, endothelin-1, endothelin-2 and endothelin-3, that exhibit potent vasoconstrictor activity have been identified. Each induces vasoconstriction with a potency order of endothelin-2 > endothelin-1 > endothelin-3. Another family of peptides, sarafotoxins, a group of peptide toxins from the venom of the snake Atractaspis eingadensis that cause severe coronary vasospasm in snake bite victims, have structural and functional homology to endothelin-1 and bind competitively to the same cardiac membrane receptors (Kloog et al. (1989) Trends Pharmacol. Sci. 10: 212-214).

The family of three isopeptides endothelin-1, endothelin-2 and endothelin-3 are encoded by a family of three genes (see, Inoue et al. (1989) Proc. Natl. Acad. Sci. USA 86: 2863-2867; see, also Saida et al. (1989) J. Biol. Chem. 264: 14613-14616). The nucleotide sequences of the three human genes are highly conserved within the region encoding the mature 21 amino acid peptides. Endothelin-2 is (Trp⁶,Leu⁷) endothelin-1 and endothelin-3 is (Thr²,Phe⁴,Thr⁵,Tyr⁶,Lys⁷,Tyr¹⁴) endothelin-1. These peptides are, thus, highly conserved at the C-terminal ends. In addition, endothelin is highly conserved among species.

Release of endothelins from cultured endothelial cells is modulated by a variety of chemical and physical stimuli and appears to be regulated at the level of transcription and/or translation. For example, gene expression of endothelin-1 is increased by adrenaline, thrombin and Ca²⁺ ionophore. The production and release of endothelin from the endothelium is stimulated by angiotensin II, vasopressin and other factors, such as endotoxin and cyclosporin (see, Brooks et al. (1991) Eur. J. Pharm. 194: 115-117), and is inhibited by nitric oxide. Endothelial cells appear to secrete short-lived endothelium-derived relaxing factors

-3-

(EDRF), such as nitric oxide or a related substance (Palmer *et al.* (1987) *Nature* 327: 524-526), when stimulated by vasoactive agents, such as acetylcholine and bradykinin. Endothelin-induced vasoconstriction is also attenuated by atrial natriuretic peptide (ANP).

- 5 The endothelin peptides exhibit numerous biological activities *in vivo* and *in vitro*. Endothelin provokes a strong and sustained vasoconstriction *in vivo* in rats and *in vitro* in isolated vascular smooth muscle preparations; it also provokes the release of eicosanoids and endothelium-derived relaxing factor (EDRF) from perfused vascular beds.
- 10 Intravenous administration of endothelin-1 and *in vitro* addition to vascular and other smooth muscle tissues produces long-lasting pressor effects and contraction, respectively (see, *e.g.*, Bolger *et al.* (1991) *Can. J. Physiol. Pharmacol.* 69: 406-413). For example, in isolated vascular strips, endothelin-1 is a potent ($EC_{50} = 4 \times 10^{-10}$ M) and slow acting, but
- 15 persistent, contractile agent. *In vivo*, a single dose elevates blood pressure in about 20 to 30 minutes. Endothelin-induced vasoconstriction is not affected by antagonists to known neurotransmitters or hormonal factors, but is abolished by calcium channel antagonists. The effect of calcium channel antagonists, however, is most likely the result of
- 20 blockage of calcium influx, since calcium influx appears to be required for the long-lasting contractile response to endothelin.

Endothelin also mediates renin release, stimulation of ANP release and induces a positive inotropic action in guinea pig atria. In the lung, endothelin-1 acts as a potent bronchoconstrictor (Maggi *et al.* (1989) *Eur. J. Pharmacol.* 160: 179-182). Endothelin increases renal vascular

25 resistance, decreases renal blood flow, and decreases glomerular filtrate rate. It is a potent mitogen of glomerular mesangial cells and invokes the phosphoinoside cascade in such cells (Simonson *et al.* (1990) *J. Clin. Invest.* 85: 790-797).

-4-

There are specific high affinity binding sites (K_d 's in the range of $2-6 \times 10^{-10}$ M) for the endothelins in the vascular system and in other tissues, including the intestine, heart, lungs, kidneys, spleen, adrenal glands and brain. Binding is not inhibited by catecholamines, vasoactive peptides, neurotoxins or calcium channel antagonists. Endothelin binds and interacts with receptor sites that are distinct from other autonomic receptors and voltage dependent calcium channels. Competitive binding studies indicate that there are multiple classes of receptors with different affinities for the endothelin isopeptides.

10 DNA clones encoding two distinct endothelin receptors, designated ET_A and ET_B , have been isolated (Arai et al. (1990) Nature **348**: 730-732; Sakurai et al. (1990) Nature **348**: 732-735). Based on the amino acid sequence of the proteins encoded by the cloned DNA, it appears that each receptor contains seven membrane spanning domains and exhibits
15 structural similarity to G-protein-coupled membrane proteins. Messenger RNA encoding both receptors has been detected in a variety of tissues, including heart, lung, kidney and brain. The distribution of receptor subtypes is tissue specific (Martin et al. (1989) Biochem. Biophys. Res. Commun. **162**: 130-137). ET_A receptors appear to be selective for
20 endothelin-1 and are predominant in cardiovascular tissues. ET_B receptors are predominant in noncardiovascular tissues, including the central nervous system and kidney, and interact with the three endothelin isopeptides (Sakurai et al. (1990) Nature **348**: 732-734). In addition, the ET_A receptors, which are endothelin-1-specific, occur on smooth muscle
25 and are linked to vasoconstriction; whereas ET_B receptors are located on the vascular endothelium and are linked to vasodilation (Takayanagi et al. (1991) FEBS Ltrrs. **282**: 103-106).

The activity of the endothelin isopeptides varies in different tissues by virtue of the distribution of receptor types and the differential affinity
30 of each isopeptide for each receptor type. For example, endothelin-1

-5-

inhibits ¹²⁵I-labelled endothelin-1 binding in cardiovascular tissues 40-700 more potently than endothelin-3. ¹²⁵I-labelled endothelin-1 binding in non-cardiovascular tissues, such as kidney, adrenal gland, and cerebellum, is inhibited to the same extent by endothelin-1 and
5 endothelin-3, which indicates that cardiovascular tissues are rich in ET_A receptors and non-cardiovascular tissues are rich in ET_B receptors.

Endothelin-1 plasma levels in healthy individuals, as measured by radioimmunoassay (RIA), are about 0.26-5 pg/ml. Blood levels of endothelin-1 and its precursor, big endothelin, are elevated in shock,
10 myocardial infarction, vasospastic angina, kidney failure and a variety of connective tissue disorders. Increased levels of circulating endothelin are present in patients with pulmonary hypertension. In patients undergoing hemodialysis or kidney transplantation or suffering from cardiogenic shock, myocardial infarction or pulmonary hypertension, endothelin levels
15 are as high as 35 pg/ml have been observed (see, Stewart et al. (1991) Annals Internal Med. 114: 464-469). The levels of endothelin at the endothelium/smooth muscle interface are probably much higher because endothelin-1 likely acts as a local, rather than a systemic, regulating factor.

20 Endothelin antagonists and antagonists

Because of the numerous physiological effects of endothelin, it appears that it has an important physiological function, and, thus, may play a critical role in some pathophysiological conditions, including asthma, hypertension, renal failure, asthma, endotoxin shock and
25 vasospasm (see, Saito et al. (1990) Hypertension 15: 734-738; Tomita et al. (1989) N.Engl.J. Med. 321: 1127; Kurihara et al. (1989) J. Cardiovasc. Pharmacol. 13(Suppl. 5): S13-S17; Morel et al. (1989) Eur. J. Pharmacol. 167: 427-428). Because endothelin is associated with these and other disease states, more detailed knowledge of the function

-6-

and structure of the endothelin peptide family should provide insight in the progression and treatment of such conditions.

Studies of structural analogs of endothelin have been conducted in order to gain insight into its role in the patho-physiology of cardiovascular disorders, such as hypertension, atherosclerosis, cerebral and coronary vasospasm, asthma and renal failure. Such studies have demonstrated the importance of the two S-S bonds, the C-terminal Trp and the cluster of charged residues Asp⁸-Lys⁹-Glu¹⁰ (see, Nishikori *et al.* (1991) Neurochem. Int. 18: 535-539) for vasoconstriction activity. Thus, compounds that can interfere with or potentiate endothelin- associated activities, such as endothelin-receptor interaction and vasoconstrictor activity, are of interest.

A limited number of compounds that exhibit endothelin antagonistic activity have been identified. In particular, a fermentation product of Streptomyces misakiensis, designated BE-18257B, has been identified as an ET_A receptor antagonist. BE-18257B is a cyclic pentapeptide, cyclo(D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp), which inhibits ¹²⁵I-labelled endothelin-1 binding in cardiovascular tissues in a concentration-dependent manner (IC₅₀ 1.4 μ M in aortic smooth muscle, 0.8 μ M in ventricle membranes and 0.5 μ M in cultured aortic smooth muscle cells), but fails to inhibit binding to receptors in tissues in which ET_B receptors predominate at concentrations up to 100 μ M. Cyclic pentapeptides related to BE-18257B, such as cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ-123), have been synthesized and shown to exhibit activity as ET_A receptor antagonists (see, U.S. Patent No. 5,114,918 to Ishikawa *et al.*; see, also, EP A1 0 436 189 to BANYU PHARMACEUTICAL CO., LTD (October 7, 1991)). Studies that measure the inhibition by these cyclic peptides of endothelin-1 binding to endothelin-specific receptors indicate that these cyclic peptides bind preferentially to ET_A receptors.

-7-

The analog [Ala^{1,3,11,15}]endothelin-1, in which the four cys residues are replaced with Ala, inhibits ¹²⁵I-endothelin-1 binding to cerebral membranes, in which ET_B receptors predominate (Hiley *et al.* (1989) Trends Pharmacol. Sci 10: 47-49). This peptide and certain truncated

5 forms of endothelin-1 elicit endothelium-dependent vasorelaxation of precontracted porcine pulmonary arteries to an extent that parallels the respective binding affinities of each form for ET_B (Saeki *et al.* (1991) Biochem. and Biophys. Res. Commun. 179: 286-292).

Endothelin antagonists and agonists as therapeutic agents

10 To aid in understanding the physiological role of endothelin, there is a need to identify compounds that modulate or alter endothelin activity. Compounds that modulate endothelin activity, particularly compounds that act as specific antagonists or agonists, may not only aid in elucidating the function of endothelin, but may be therapeutically useful.

15 In particular, compounds that specifically interfere with the interaction of endothelin peptides with the ET_A, ET_B or other receptors should aid in the design of therapeutic agents, and may be useful as disease specific therapeutic agents.

In addition, compounds that specifically interfere with binding of

20 endothelin peptides to ET_A or ET_B receptors should be useful in identifying essential characteristics of such agents and as disease specific therapeutic agents. Many of the endothelin activity-modulating compounds that have been identified are peptides. Peptides tend to undergo extensive enzymatic degradation *in vivo* resulting in loss of

25 bioactivity. Thus, such compounds may not be therapeutically effective and, certainly, are not ideally suited for pharmaceutical use. There is, therefore, a need for the identification of compounds that can act to modulate endothelin activity, particularly compounds that act as endothelin antagonists and agonists, but that are smaller than peptides

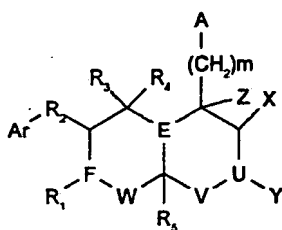
-8-

and/or that are non-peptidic in nature and that are thereby more suitable for pharmaceutical use.

Therefore, it is an object herein to provide non-peptide compounds or compounds that are not solely composed of amino acids and peptide linkages and that modulate the activity of one or more of the endothelin isopeptides. It is another object herein, to provide compounds that have activity as specific endothelin antagonists. It is also an object herein to provide compounds that are useful for treatment of disorders that are mediated by the action of endothelin. It is also an object herein to provide compounds that specifically interact with or inhibit the interaction of endothelin peptides with ET_A, ET_B receptors or other ET receptors. It is also an object herein to provide methods for distinguishing between ET_A and ET_B receptors, for identifying endothelin-specific receptors, and for purifying such endothelin-specific receptors.

SUMMARY OF THE INVENTION

Compounds of formula (I):

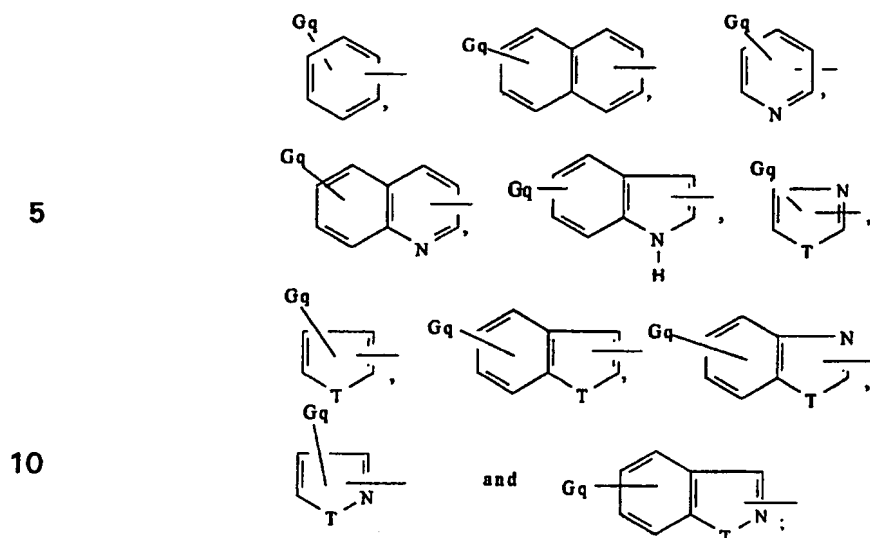


in which:

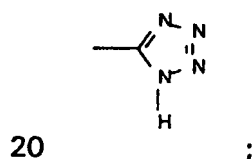
Ar is a substituted or unsubstituted aromatic or heteroaromatic group, including, but not limited to:

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-9-



A is a functional group that bears a polar moiety and includes, but is not limited to: COOH, SO₃H, PO₃H, OH, NHR, CONHR, CON(R)₂,
 15 CONHSO₂R₁, SO₂NHCOR₁, CONHCOR₁ or tetrazole, which has the formula:



R₁ is R, R-C=O, R substituted with one or more heteroatoms, including, but not limited to O, N, S, P, halogen, CN, N₃ or NO₂, or a substituted or unsubstituted aryl group, or is aryl-(CH₂)_n, in which the aryl group is preferably a lower aryl group and most preferably is Ar;

25 R is H or is selected from the following groups that have from 1-20 carbon atoms, preferably 1-10 carbon atoms, more preferably 1-8 carbon atoms and most preferably 1-6 carbon atoms: a substituted or unsubstituted straight branched or cyclic alkyl, alkenyl, aryl or alkynyl group; a mixture of branched or cyclic alkyl, alkenyl, and alkynyl groups;
 30 an aryl alkyl group an alkoxyalkyl group; and an alkoxycarbonyl group;

-10-

R_2 , which is selected independently from R_1 , is $(CH_2)_n$, CHR, $C(R)_2$, COO, OCO, OCH_2 , NHCO, CONH, SO, SO_2 or NR;

R_3 and R_4 , which are the same or different or, independent of the other each may be absent, are =O, H, O-aryl, OR, O-alkyl or alkyl, aryl, SR, S-aryl, NHR, NH-aryl, NR, or other heteroaromatic groups, in which the alkyl and aryl groups are preferably lower alkyl or lower aryl and the aryl groups are preferably Ar;

R_5 is H, OH or R;

E and F, which are the same or are different, are N, $(CH_2)_p$, NR, or but preferably N or $(CH_2)_p$, and more preferably N or $(CH_2)_p$ such that at least one of E and F is N;

G_q , which is selected independently from R_1 and R_3 , is R_1 , R_3 halogen, CN, NO_2 or N_3 ;

T is O, S, NR, or NCOR;

U and V, which may be the same or different, are $(CH_2)_n$;

W is CO, $C(R)_2$, $(CH_2)_n$, $(CH_2)_n$ -CHR or CHR- $(CH_2)_n$;

X and Y, which may be the same or different, are H, alkyl or aryl or X and Y form a saturated or unsaturated, substituted or unsubstituted, homocyclic or heterocyclic ring containing 3-15 members, preferably 5-9 members;

Z is H, OH, OR, SR, NHR or $N(R)_2$; and

p is an integer or 0 between 0 and 5, inclusive, and is preferably between 0 and 3, inclusive;

q is an integer or zero between 0 and 5, inclusive;

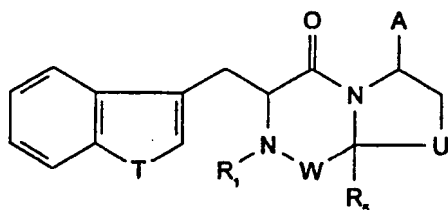
m and n are integers or 0 between 0 and 10, inclusive, and are preferably between 0 and 4, inclusive;

n is selected independently for each of R, R_1 , R_2 , U, V and W;

n, m, p and q, may be the same or different, are provided.

In particular compounds of formula (I) that have formula (II) are preferred:

-11-



(II)

5

in which:

A is CO_2H , COH , CO-alkyl or CONH_2 , in which the alkyl group is preferably an alkyl group having 1-10 carbon atoms and most preferably a lower alkyl group;

10 W is CH_2 , CH_2CHOH , or CO ;

n is an integer or 0 between 0 and 4, inclusive; and

m is 0.

Other preferred compounds include those of formula II in which:

R_1 is as defined above, but is preferably an alkyl group, more

15 preferably an alkyl group having 1-10 carbon atoms and most preferably a lower alkyl group;

E and F are N; and

T is NH , NCH_3 , NCH_2CH_3 , or NCOR .

20 Compositions containing therapeutically effective concentrations of the compounds of formula I formulated for oral, intravenous, local and topical application for the treatment of hypertension, cardiovascular diseases, cardiac diseases, including myocardial infarction, respiratory diseases, including asthma, inflammatory diseases, ophthalmologic diseases, gastroenteric diseases, renal failure, endotoxin shock, anaphylactic shock, hemorrhagic shock, and other diseases in which endothelin mediated physiological responses are implicated are provided.

25 Methods of treatment of hypertension, cardiovascular diseases, cardiac diseases including myocardial infarction, respiratory diseases, including asthma, inflammatory diseases, ophthalmologic diseases, 30 gastroenteric diseases, renal failure, endotoxin shock, anaphylactic

-12-

shock, hemorrhagic shock, and other diseases in which endothelin mediated physiological responses are implicated. using effective amounts of the compositions are also provided.

- In practicing the methods, effective amounts of compositions
- 5 containing therapeutically effective concentrations of the compounds formulated for oral, intravenous, local and topical application for the treatment of hypertension, cardiovascular diseases, cardiac diseases, including myocardial infarction, respiratory diseases, including asthma, inflammatory diseases, ophthalmologic diseases, gastroenteric diseases,
- 10 renal failure, endotoxin shock, anaphylactic shock, hemorrhagic shock, and other diseases in which endothelin mediated physiological responses are implicated are administered to an individual exhibiting the symptoms of one or more of these disorders. The amounts are effective to ameliorate or eliminate one or more symptoms of the disorders.
- 15 Methods for detecting, distinguishing and isolating endothelin receptors using the compounds of formula (I) are also provided. In addition, methods for screening compounds for use for treating particular diseases based on their preferential affinity for a particular endothelin receptor subtype are provided.

20 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

- Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications
- 25 referred to herein are incorporated by reference herein.

- As used herein, a peptidomimetic is a compound that mimics the conformation and certain stereochemical features of the biologically active form of a particular peptide. In general, peptidomimetics are designed to mimic certain desirable properties of a compound but not the
- 30 undesirable features, such as flexibility leading to a loss of the biologically

-13-

active conformation and bond breakdown. For example, methylenethio bioisostere [CH₂S] has been used as an amide replacement in enkephalin analogs (see, e.g., Spatola, A.F. Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins (Weistein, B, Ed.), Vol. 7, pp. 267-357,

- 5 Marcel Dekker, New York (1983); and Szelke et al., In Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium, (Hruby and Rich, Eds.); pp. 579-582, Pierce Chemical Co., Rockford, Illinois (1983)).

A peptidomimetic is, thus, a compound that mimics certain
10 properties of a peptide. For example, morphine is a compound which can be orally administered, and which is a peptidomimetic of the peptide endorphin.

As used herein, endothelin peptides include peptides that have substantially the amino acid sequence of endothelin-1, endothelin-2,
15 endothelin-3 and that act as potent endogenous vasoconstrictor peptides.

As used herein, endothelin antagonists include compounds that inhibit endothelin-stimulated vasoconstriction and contractions and other endothelin-mediated physiological responses. The antagonist may act by interfering with interaction of the endothelin with an endothelin-specific
20 receptor or by interfering with the physiological response or bioactivity of endothelin, such as vasoconstriction. The effectiveness of a potential antagonist can be assessed using methods known to those of skill in the art. For example, the properties of potential antagonist may be assessed as a function of its ability to inhibit an endothelin induced activity in vitro
25 using a particular tissue, such as rat portal vein and aorta as well as rat uterus, trachea and vas deferens (see e.g., Borges, R., Von Grafenstein, H. and Knight, D.E. (1989) Tissue selectivity of endothelin, Eur. J. Pharmacol 165:223-230). The ability to act as an endothelin antagonist in vivo can be tested in hypertensive rats, ddY mice or other recognized
30 animal model (see, Kaltenbronn et al. (1990) J. Med. Chem. 33:838-845,

-14-

see, also U.S. Patent No. 5,114,918 to Ishikawa et al.; EP A1 0 436 189 to BANYU PHARMACEUTICAL CO., LTD (October 7, 1991); and Bolger et al. (1983) J. Pharmacol. Exp. Ther. 225:291-309).

As used herein, an endothelin antagonist interferes with endothelin-
5 stimulated vasoconstriction or that competitively inhibit binding of
endothelin to particular receptors, such as ET_A receptors, as assessed by
assays known to those of skill in the art. For example, endothelin activity
can be identified by the ability of endothelin to stimulate vasoconstriction
of isolated rat thoracic aorta or portal vein ring segments (Borges et al.
10 (1989) "Tissue selectivity of endothelin" Eur. J. Pharmacol. 165: 223-
230). To perform the assay, the endothelium is abraded and ring
segments are mounted under tension on stirrups in a tissue bath and
exposed to endothelin in the presence of the test compound or
compounds. Changes in endothelin-induced tension are recorded. Dose
15 response curves may be generated and used to provide information
regarding the relative potency of the test compound. Other tissues that
may be used for evaluating the effects on tissue contraction include
heart, skeletal muscle, kidney, uterus, trachea and vas deferens.
Endothelin receptor isotype specific antagonist compounds may be
20 identified by the ability of such compounds to interfere with endothelin
binding to different tissues or cells expressing specific endothelin-receptor
subtypes, or to interfere with biological effects of endothelin or an
endothelin isotype, thereby exhibiting endothelin-receptor subtype
specificity. For example, ET_B receptors are expressed in vascular
25 endothelial cells, possibly mediating the release of prostacyclin and
endothelium-derived relaxing factor (De Nucci et al. (1988) Proc. Natl.
Acad. Sci. USA 85:9797). ET_A receptors are not detected in cultured
endothelial cells, which express ET_B receptors. For example, the binding
of compounds or inhibition of binding of endothelin to ET_B receptors can
30 be assessed by measuring the inhibition of endothelin-1-mediated release

-15-

of prostacyclin, as measured by its major stable metabolite, 6-keto PGF_{1 α} , from cultured bovine aortic endothelial cells (see, e.g., Filep et al. (1991) Biochem. and Biophys Res. Commun. 177: 171-176).

Thus, the relative affinity of the compounds for different endothelin
5 receptors may be evaluated by determining dose response curves to using tissues that differ in receptor subtype.

As used herein, the biological activity or bioactivity of endothelin includes any activity induced, potentiated or influenced by endothelin in vivo. It also includes the ability to bind to particular receptors and to
10 induce a functional response, such as vasoconstriction. These activities, which appear to be a function of the receptor subtype expressed in a particular tissue, include, but are not limited to, vasoconstriction, vasorelaxation and bronchodilation. For example, ET_B receptors appear to be expressed in vascular endothelial cells and may mediate vasodilation
15 and other such responses; whereas ET_A receptors, which are endothelin-1-specific, occur on smooth muscle and are linked to vasoconstriction. Any assay known to those of skill in the art to measure or detect such activity may be used to assess such activity (see, e.g., Spokes et al. (19989) J. Cardiovasc. Pharmacol. 13(Suppl. 5):S191-S192; Spinella et al. (1991) Proc. Natl. Acad. Sci. USA 88: 7443-7446; Cardell et al.
20 (1991) Neurochem. Int. 18:571-574); and the Examples herein).

As used herein, the IC₅₀ refers to 50% of inhibition of the maximal response, such as binding of endothelin to tissue receptors.

As used herein, EC₅₀ refers to 50% of maximal expression of a
25 particular response induced, provoked or potentiated by a particular test compound.

As used herein, a lower alkyl or lower alkenyl refers to a carbon chain that contains six or fewer carbons. Such chains may be branched, straight, cyclic or any combination of branched, straight and cyclic.

-16-

As used herein, a non-peptidic compound refers to compounds that do not include more than two linked amino acids and that include linkages other than peptide bonds among the constituent groups.

As used herein, biological activity refers the in vivo activities of a
5 compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutical effects and pharmaceutical activity of such compounds, compositions and mixtures.

As used herein, a prodrug is a compound that, upon in vivo
10 administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound.

As used herein, lower alkyl, alkenyl, alkynyl, alkoxyalkyl or alkoxy carbonyl groups refer to substituents groups that have from 1-6 carbon atoms.

15 As used herein an effective amount of a compound for treating a particular disease, is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure
20 the disease but, typically is administered in order to ameliorate the symptoms of the disease. Typically repeated administration is required to achieve the desired amelioration of symptoms.

As used herein, definitions of substituents are limited to forms that could, by virtue of the principles of chemistry, exist. Thus, unless
25 otherwise specified, it is implicit that carbon has four bonds, whether single, double or conjugated, in any of the compounds encompassed by the formulas set forth herein.

As used herein, the abbreviations for amino acids and protective group are in accord with their common usage and the IUPAC-IUB
30 Commission on Biochemical Nomenclature (see, (1972) Biochem. 11:

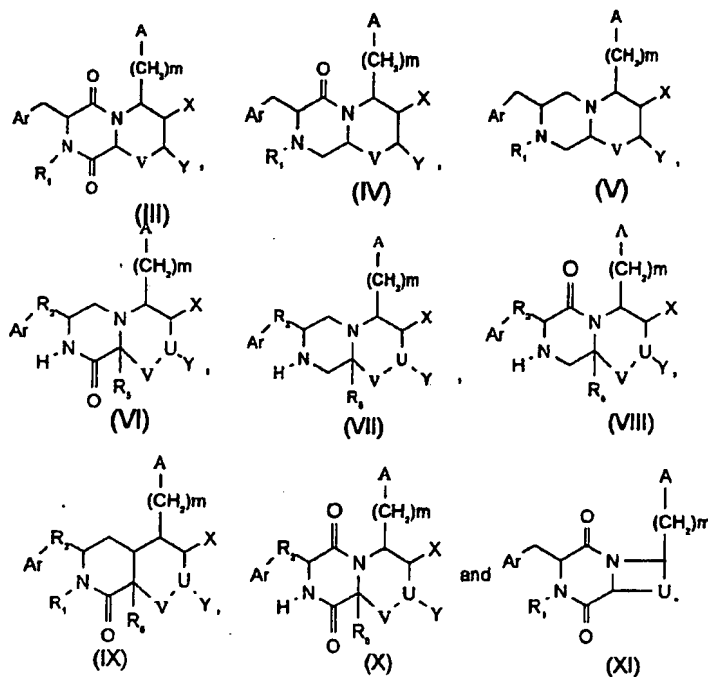
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1726). Each amino acid is identified by the standard three letter code for the naturally occurring L-amino acids; the prefix "D" indicates that the stereoisomeric form of the amino acid is D.

Description of the compounds that modulate endothelin activity

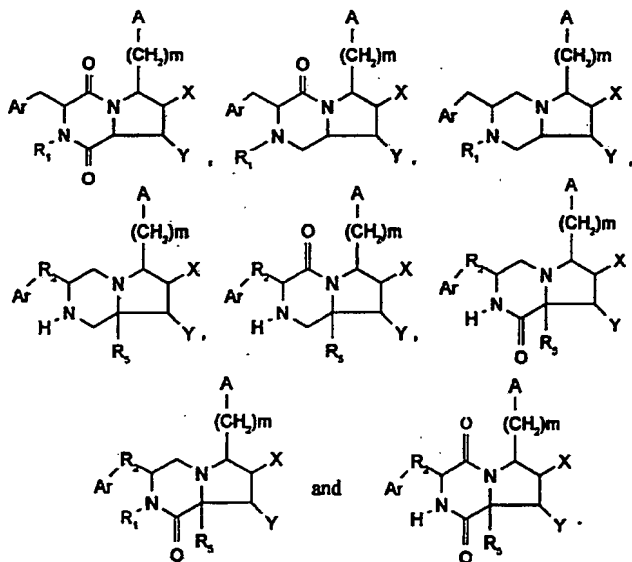
- 5 Compounds that contain two or more fused substituted or unsubstituted, saturated or unsaturated heterocyclic rings that modulate the *in vivo* or *in vitro* activity of at least one endothelin isopeptide are provided. More particularly, compounds of formula I, defined above, and pharmaceutically acceptable salts, esters and prodrugs thereof are
- 10 provided.

More preferred compounds of formula (I) are those with formulas III-XI:



-18-

Other preferred compounds include the following
bicyclo[4.3.0]nonanes:



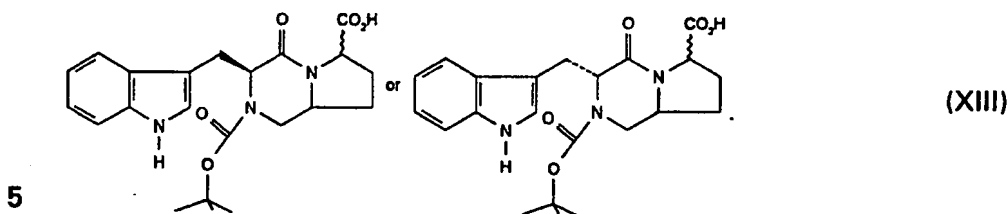
More preferred compounds are the bicyclo[4.3.0]nonanes of
5 formulas III-XI in which T is NCH_3 or NH , m is 0 or 1, A is COOH , when
m is 0 or 1, or is OH , when m is 1, and X, Y and R are H. Of these the
more preferred compounds are those in which Ar is



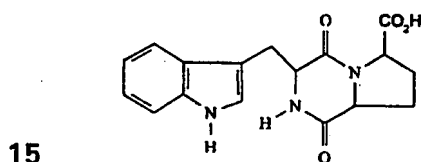
More preferred of these bicyclo[4.3.0]nonanes are those in which R_1 is 2,
2-diphenylethyl, A is COOH , m is 0, X and Y are H.

Other preferred compounds include stereoisomers of 3-(3-
15 indolylmethyl)-1,4-diaza-2-oxobicyclo[4.3.0]nonane-9-carboxylic acid,
including the stereoisomers that have the following formulae XIII:

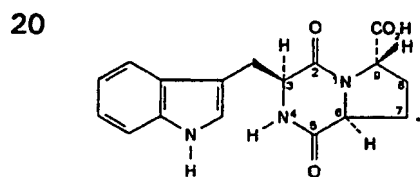
-19-



Other preferred compounds include those of formula I that have
 formula II, defined above. A particularly preferred compound is 3-(3-
 10 indolylmethyl)-1,4-diaza-2,5-dioxo-bicyclo[4.3.0]nonane-9-carboxylic acid
 of formula XIV:



Most preferred compounds include pharmaceutically active stereo-
 isomers of formula XIV, including a stereoisomer having a structure
 represented by formula XV:



25 Various stereoisomers of the compounds of formulas XII and XIV
 have been synthesized, purified and tested for activity as ET_A and/or ET_B
 agonists or antagonists.

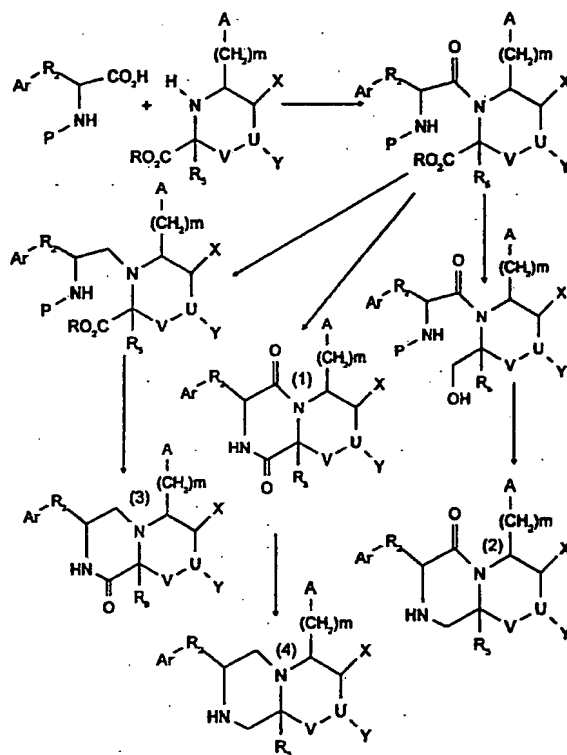
Methods for synthesis of the compounds that modulate endothelin activity

30 The compounds of formula (I) may be prepared by any suitable
 synthetic scheme. Such schemes should be apparent to one of skill in

-20-

this art. For example, the compounds may be synthesized by hydrogenation of the corresponding aromatic or heteroaromatic compounds which can be synthesized by standard methods (see, e.g., Hydrogenation Methods Rylander, Academic Press, New York, 1990 and

- 5 Comprehensive heterocyclic Chemistry Katritzky et al. eds., Pergamon Press, Oxford, 1980). In particular, the piperazines can be synthesized from the corresponding protected α -amino acids and N-(2-carboalkoxy)-alkyl- α -amino acids according to the following processes, which are represented schematically below:



- 10 (1) Synthesis of the compounds of formula (I) in which E and F are N, W is C=O and R₃ is absent and R₄ is =O

The piperazine-2,5-diones can be prepared by coupling an N-protected α -amino acid with an N-(2-carboalkoxy)alkyl α -amino acid ester under standard, well known, peptide coupling conditions to form the

-21-

corresponding dipeptide which is then deprotected and cyclized to the produce the compounds described herein.

(2) Synthesis of the compounds of formula (I) in which E and F are N, W is CH₂, CHR or RCR, R₃ is absent and R₄ is =O

5 To produce the compounds of formula (I) in which E and F are N, W is CH₂, CHR or RCR, R₃ is absent and R₄ is =O, the intermediate amide ester can be reduced to the amide alcohol using suitable reducing agents, including, but not limited to, sodium borohydride and diisobutylaluminum hydride, and then cyclized to give 2-piperazinones.

10 **(3) Synthesis of the compounds of formula (I) in which E and F are N, W is =O, R₃ and R₄ are, independently, H or R**

To produce the compounds of formula (I) in which E and F are N, W is =O, R₃ and R₄ are H or R, the intermediate amides can be reduced to the amine with reducing agents, such as, but not limited to, diborane.

15 Subsequent cyclization as described in (1) can produce 5-piperazinones.

(4) Synthesis of the piperazine compounds of formula (I) in which E and F are N and W is CH₂

The piperazines can be produced by reduction of the piperazine-2,5-diones ((1), above) with diborane, aluminum hydride or lithium

20 aluminum hydride. Introduction of R₁ at N-4 in these compounds may be effected by alkylation or acylation reactions known to those skilled in the art of organic synthesis to produce the corresponding 4-substituted compounds.

25 **(5) Synthesis of stereoisomers of the compounds of formula (I) in which E and F are N and W is CH₂ and R₁ is for tert-butoxycarbonyl (BOC)**

These compounds may be synthesized by the protocol set forth in detail in the Examples.

30 **(6) Isolation of stereoisomers in which E and F are N and W is CH₂ and R₁ is H and T is HN or CH₃NH**

Stereoisomers may be isolated using standard methods, including HPLC, column chromatograph, and fractional crystallization, known to

-22-

those of skill in this art. Synthesis and isolation of such stereoisomers is set forth in the Examples.

Pharmaceutically acceptable salts, esters of the compounds provided herein may be prepared by any suitable method known to those of skill in this art. Stereoisomers may be separated by methods, such as recrystallization and high performance liquid chromatography (HPLC) separation, known to those of skill in this art (for a review of numerous procedures, see, e.g., Jacques *et al.* (1981) Enantiomers, Racemates, and Resolutions, John Wiley & Sons, New York).

10 Description of compositions containing the compounds

Pharmaceutical compositions containing therapeutically effective concentrations of at least one of the compounds of formula I, salts or esters thereof, in a pharmaceutically acceptable carrier are provided.

The concentrations of compounds provided herein include those that are therapeutically effective for treatment of endothelin-mediated diseases. Such disorders include, but are not limited to, hypertension, cardiovascular diseases, cardiac diseases including myocardial infarction, respiratory diseases, including asthma, inflammatory diseases, ophthalmologic diseases, gastroenteric diseases, renal failure, endotoxin shock, anaphylactic shock, hemorrhagic shock, and other diseases in which endothelin mediated physiological responses are implicated.

Particularly preferred compositions are effective for the amelioration of the symptoms of hypertension or endotoxin shock.

Evaluation of the bioactivity of the compounds

After synthesis, the bioactivity of the compounds may be evaluated. Standard physiological, pharmacological and biochemical procedures are available for testing the compounds to ascertain whether the compound possess any biological activities of an endothelin peptide or the ability to interfere with, inhibit or potentiate the activity of endothelin peptides.

-23-

Screening compounds for the ability to modulate the activity of an endothelin peptide

Numerous assays are known to those of skill in the art for evaluating the ability of compounds to modulate the activity of endothelin

5 (see, e.g., U.S. Patent No. 5,114,918 to Ishikawa et al.; EP A1 0 436 189 to BANYU PHARMACEUTICAL CO., LTD (October 7, 1991); Borges et al. (1989) Eur. J. Pharm. **165**: 223-230; Filep et al. (1991) Biochem. Biophys. Res. Commun. **177**: 171-176). HERE For example, assays that assess the induction or inhibition of the contractile response of

10 thoracic aorta tissue to endothelin-1 or endothelin-1 analogs, antagonists and agonists may be used to detect compounds that exhibit antagonist or agonist activity (see, Borges et al. (1989) Eur. J. Pharmacol. **165**: 223-230). The binding of compounds or inhibition of binding of endothelin to ET_B receptors can be assessed by measuring the inhibition of endothelin-

15 1-mediated release of prostacyclin, as measured by its major stable metabolite, 6-keto PGF_{1 α} , from cultured bovine aortic endothelial cells (see, e.g., Ogawa et al. (1991) Biochem. and Biophys Res. Commun. **178**: 248-255).

In vitro studies may be corroborated with in vivo animal studies

20 (see, U.S. Patent No. 5,114,918 to Ishikawa et al.; see, also, EP A1 0 436 189 to BANYU PHARMACEUTICAL CO., LTD. (October 7, 1991)). Effective dosages for treatment of animals, including humans, may be extrapolated from in vitro and/or in vivo data (see, e.g., Bolger et al. (1983) J. Pharmacol. Exp. Ther. **225**:291-309 and U.S. Patent No.

25 5,114,918 to Ishikawa et al.).

Using such assays, the relative affinities of the compounds for ET_A receptors and ET_B receptors may be assessed and those that possess the desired properties, such as specific inhibition of binding of endothelin-1, may be selected. The selected compounds that exhibit activities that

30 may be therapeutically useful may be formulated in suitable pharmaceutical compositions.

-24-

Formulation of pharmaceutical compositions

Pharmaceutical carriers or vehicles suitable for administration of the compounds provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

- 5 In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients.

- The active compounds can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally,
10 subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner suitable for each route of administration.

The active compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of serious toxic effects on the patient treated.

- 15 The therapeutically effective concentration may be determined empirically by testing the compounds in known in vitro and in vivo systems (see, e.g., U.S. Patent No. 5,114,918 to Ishikawa et al.; EP A1 0 436 189 to BANYU PHARMACEUTICAL CO., LTD (October 7, 1991); Borges et al. (1989) Eur. J. Pharm. 165: 223-230; Filep et al. (1991)
20 Biochem. Biophys. Res. Commun. 177: 171-176).

The concentration of active compound in the drug composition will depend on absorption, inactivation, excretion rates of the active compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

- 25 Typically a therapeutically effective dosage should produce a serum concentration of active ingredient of from about 0.1 ng/ml to about 50-100 μ g/ml/kg body weight. The pharmaceutical compositions typically should provide a dosage of from about 0.01 to about 50 mg of compound per kilogram of body weight per day. The active ingredient
30 may be administered at once, or may be divided into a number of smaller

-25-

doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be
5 noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of
10 the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

If oral administration is desired, the compound should be provided in a composition that protects it from the acidic environment of the
15 stomach. For example, the composition can be formulated in an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. The composition may also be formulated in combination with an antacid or other such ingredient.

Oral compositions will generally include an inert diluent or an edible
20 carrier and may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the
25 composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose, a disintegrating agent such as, but
30 not limited to, alginic acid and corn starch; a lubricant such as, but not

-26-

limited to, magnesium stearate; a glidant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; and a flavoring agent such as peppermint, methyl salicylate, and fruit flavoring.

- 5 When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The compounds can also be administered
- 10 as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

- The active materials can also be mixed with other active materials
- 15 which do not impair the desired therapeutic effect or with materials that supplement the desired action, such as, for example, if the compound is used for treating asthma or hypertension, with other bronchodilators and antihypertensive agents, respectively.

- Solutions or suspensions used for parenteral, intradermal,
- 20 subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite;
- 25 chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parental preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

-27-

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. For example, liposome formulations may be prepared as described in U.S. Patent No. 4,522,811.

The active compounds may be prepared with carriers that protect the compound against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as collagen, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others. Methods for preparation of such formulations are known to those skilled in the art.

The compounds may be formulated for local or topical application, such as for topical application to the skin in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Such solutions, particularly those intended for ophthalmic use, may be formulated as 0.01% - 10% isotonic solutions, pH about 5-7, with appropriate salts. The compounds may be formulated as aerosols for topical application, such as by inhalation (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923, which describe aerosols for delivery of a steroid useful for treatment inflammatory diseases, particularly asthma).

Preferred compositions are formulated for oral or intravenous administration for the treatment of hypertension or endotoxin shock.

-28-

Affinity isolation of endothelin receptors

The compounds may be used in methods for identifying and isolating endothelin-specific receptors. One or more of the compounds may be linked, covalently or by other linkage, to an appropriate resin or other support, such as Affi-gel, by methods known to those of skill in the art, such as methods in which like compounds are bound to such resins (see, e.g., Schwartz *et al.* (1990) Endocrinology 126: 3218-3222). The selected compound or compounds may be one or those that is (are) specific for E_A or E_B receptors or other subclass of receptor. The resin is pre-equilibrated with a suitable buffer at a physiological pH (pH 7-8). A composition containing solubilized receptors from a selected tissue are contacted with the resin to which the compound is bound and the receptors are selectively eluted and identified by testing them for binding to an endothelin peptide. Preparation of the receptors and resin and elution may be performed by modification of standard protocols known to those of skill in the art (see, e.g., Schwartz *et al.* (1990) Endocrinology 126: 3218-3222).

Other methods for distinguishing receptor type based on differential affinity to any of the compounds provided herein are provided. Any of the assays described herein for measuring the affinity of selected compounds for endothelin receptors may also be used to distinguish receptors subtypes based on affinity for particular compounds provided herein. In particular, ET_A and ET_B receptors may be identified by measuring the binding affinity of the unknown receptor for a compound provided herein that has a known affinity for one receptor over the other. Such preferential interaction is useful for determining the particular disease that may be treated with a compound prepared as described herein. For example, compounds with high affinity for ET_A receptors and little or no affinity for ET_B receptors are candidates for use

-29-

as hypertensive agents; whereas, compounds that preferentially interact with ET_B receptors are candidates for use as anti-asthma agents

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

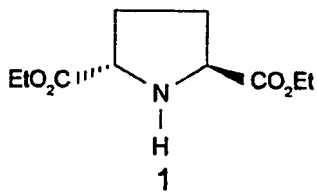
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EXAMPLE 1**PREPARATION OF (3R,6R,9R) and (3R,6S,9S)-3-(3-INDOLYLMETHYL)-1,4-DIAZA-2,5-DIOXO-BICYCLO[4.3.0]NONANE-9-CARBOXYLIC ACID****A. (2R*,5R*)-Dicarbethoxypyrrolidine (1)**

- 1.7g (27 mmol) of ammonium formate and 0.17g of 10% Pd/C were added to a solution of 1.02g (3.34 mmol) of (2R*,5R*)-dicarbethoxy-N-benzylpyrrolidine in 5.0 ml of dry ethanol. The resulting black suspension was stirred under an atmosphere of argon at reflux temperature for a period of 8 hr during which gases evolved. Stirring was then discontinued and the reaction mixture was filtered through a pad of Celite and washed with ethanol. The ethanol was removed in vacuo and the remaining suspension was dissolved in 40 ml of ethyl acetate, washed with water (2x5ml) and brine (1x5ml), and dried over anhydrous $MgSO_4$. Concentration in vacuo produced 0.72g (76.6%) of a clear colorless oil that was homogenous as evidenced TLC (R_f 0.42, 1:2 EtOAc/Hexane) and that had the following 1H NMR data: (CDCl₃, 360MHZ) spectroscopic data:

δ 4.09-4.29 (4H,m) 3.94-4.06 (2H,m), 2.83-3.03 (1H,br s), 2.59-2.68 (2H,m), 1.89-2.39 (2H,m), 1.21-1.43 (6H,t).

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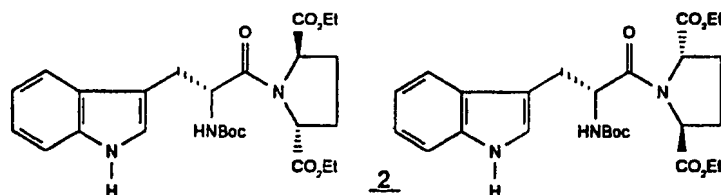
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B. (2R*,5R*)-N-Boc-R-Tryptophanyl-2,5-dicarbethoxypyrrolidine (2)

3.9 g (19 mmol) of 1,3-dicyclohexylcarbodiimide (DCC) was added at 0° C to a solution containing 1.63g (7.58 mmol) of (1), 2.54g (11 mmol) of Boc-D-tryptophan, 2.56 g (19 mmol) of 1-hydroxybenzotriazole hydrate and 3.3 ml (19 mmol) of diisopropylethylamine in 25 ml of dry dimethylformamide (DMF). The resulting clear colorless solution was stirred under an argon atmosphere at room temperature for a period of 18 h, resulting in the formation of a white precipitate. TLC analysis of the mixture (97:3 CHCl₃/MeOH) indicated that there was some remaining pyrrolidine diester. Therefore, an additional 2.0 g of DCC was added and the solution was stirred for an additional 5 h at room temperature. Ethyl acetate (80ml) was added to the reaction mixture and the solution was washed with water (3x25ml) and brine solution (1x25ml) and dried over MgSO₄. Solvents were removed in vacuo to produce 4.53g of a thick yellow oil. Chromatography of the crude product on silica gel using the eluent showed that the product was a diastereomeric mixture (R_f=0.29 and 0.25) of the coupled products in an approximate ratio of 1.5:1. Total yield for both of the fractions containing the tryptophan pyrrolidine diester was 1.69 g (44.6%). The diastereomer possessing the higher R_f(0.29) was separated and analyzed yielding the following ¹H NMR (CDCl₃, 360MHz) spectroscopic data:

δ 8.04-8.11 (1H,br s), 7.73-7.79 (1H,d), 7.32-7.38 (1H,d), 7.10-7.24 (3H,m), 5.27-5.34 (1H,d), 4.54-4.68 (2H,m), 4.05-4.28 (4H,m), 3.90-3.97 (1H,d), 3.13-3.22 (2H,d), 1.36-1.42 (4H,m), 1.18-1.42 (m,15H)

-31-



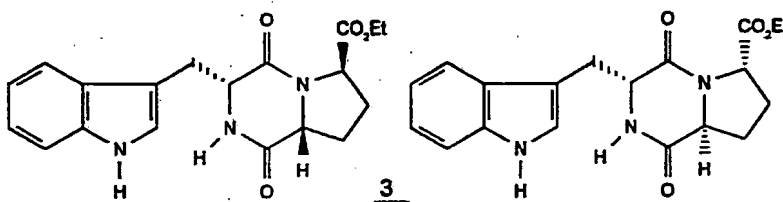
C. Methyl (3R,6R,9R) and (3R,6S,9S)-3-(3-Indolylmethyl)-1,4-diaza-2,5-dioxobicyclo [4.3.0] nonane-9-carboxylate (3)

- A 25% solution of TFA in CH_2Cl_2 (25 ml) was added at 0°C to an
- 5 isomeric mixture 0.94g (1.9 mmol) of the tryptophan pyrrolidine diester (2) and 0.41 (3.8 mmol) anisole. The reaction mixture was stirred at 0°C for 15 min and then at room temperature for 2 h. TLC analysis (93:7, $\text{CHCl}_3/\text{MeOH}$) using a ninhydrin indicator showed the complete
- 10 disappearance of the starting material and the appearance of a new spot near the baseline. Without isolation of the product, the TFA was neutralized by the addition of 12.12 ml of diisopropylethylamine and the mixture turned from dark red to yellow. After stirring at room
- temperature for approximately 6 h, TLC analysis of the reaction mixture (95:5; $\text{CHCl}_3/\text{MeOH}$) showed the presence of two major isomers in
- 15 approximately a 2:1 ratio. After removal of the CH_2Cl_2 in vacuo, the suspension was extracted into ethyl acetate and washed and dried over anhydrous MgSO_4 . Concentration in vacuo resulted in 0.88g of a dark brown oil. Column chromatography over silica gel (CHCl_3) produced
- 0.30g (44%) of the more mobile isomer (3a) that yielded the following ^1H
- 20 NMR (CDCl_3 , 360 MHz) data:
- δ 8.18-8.34 (1H,br s), 7.48-7.62 (1H,d), 7.29-7.43 (1H,d), 7.00-7.28 (3H,m), 5.66-5.83 (1H,s), 4.51-4.63 (1H,t), 4.38-4.50 (1H,m), 4.12-4.43 (3H,m), 3.62-3.78 (1H,m), 2.86-3.02 (1H,m), 2.23-2.43 (2H,m), 1.83-2.12 (2H,m), 1.24-1.36 (3H,t).

-32-

Further elution with chloroform gave 0.15 g of the less mobile isomer (**3b**) (m.p. 144-149 ° C) that had the following ¹H NMR (CDCl₃, 360 MHz) spectroscopic data:

5 8.19-8.39 (1H,br s), 7.49-7.61 (1H,d), 7.29-7.43 (1H,d), 7.01-7.28 (3H,m), 6.21 (1H,br s), 4.62 (1H,t), 4.23-4.34 (3H,m), 3.81-3.93 (1H,m), 1.73-1.05 (2H,m), 1.25-1.36 (3H,t).



D. (3R,6R,9R) and (3R,6S,9S)-3-(3-indolylmethyl)-1,4-diaza-2,5-dioxo-bicyclo[4.3.0]nonane-9-carboxylic acid (4**)**

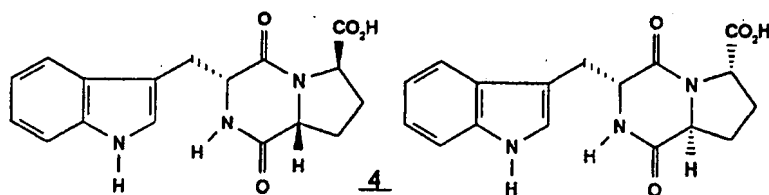
10 A 1N NaOH solution (90 μ l, 0.09 mmol) was added to a solution of 0.032g (0.09 mmol) of (**3a**) dissolved in 3.0 ml of methanol-water solution. The clear solution was stirred at room temperature for approximately 3 hr. TLC analysis indicated that only a trace of unreacted starting material (95:5; CHCl₃/MeOH) was present. Ethyl acetate (2.0 ml)

15 was added to the solution and the mixture was transferred to a separatory funnel. Following dilution with about 4 ml of water, two layers separated. The aqueous layer was then acidified to about pH 2 with 0.05 N HCl when brown oil droplets fell out of the aqueous layer and extracted with ethyl acetate (3x20ml). The extract was dried over

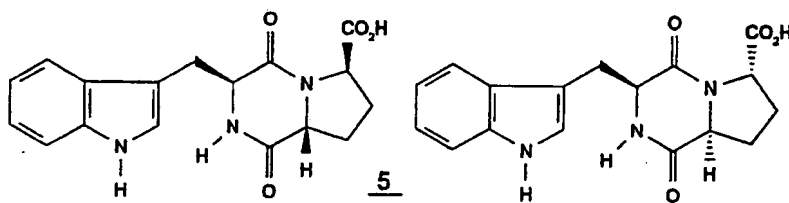
20 MgSO₄. Concentration of the solvents in vacuo produced 18.6 mg (63.9%) of a crude product that appeared as a semi-white crystalline powder.

TLC of this material (85:10:5; CHCl₃/MeOH/AcOH) showed two major spots (R_f =0.17 and 0.22).

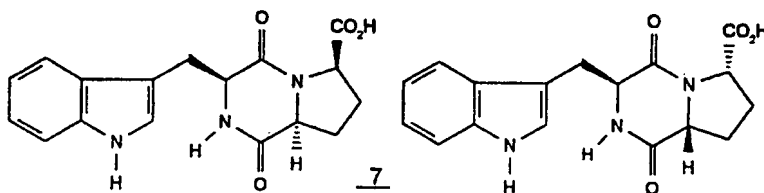
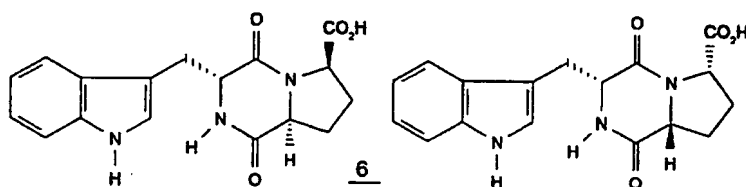
-33-



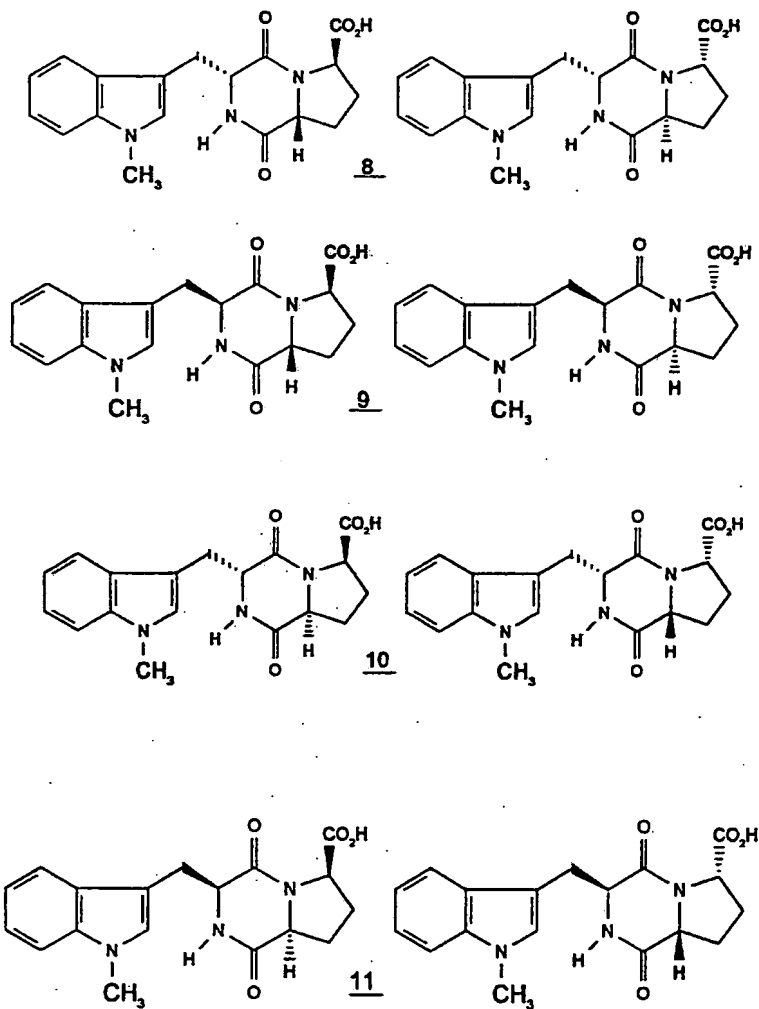
Following the above protocol, but using alternative stereoisomers and reagents, stereoisomers (formulas 5 - 11) of 3-(3-indolylmethyl)-1,4-diaza-2,5-dioxobicyclo [4.3.0] nonane-9-carboxylic acid have been synthesized.



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-34-



EXAMPLE 2

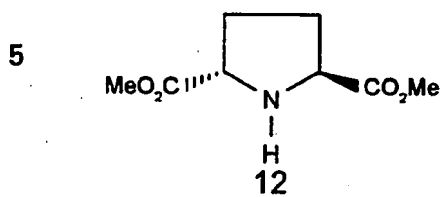
PREPARATION OF (3R,6R,9R) AND (3R,6S,9S)-2-OXO-3 (3-INDOLYLMETHYL)-4-*TERT*-BUTOXYCARBONYL-1,4-DIAZOBI-CYCLO[4.3.0]NONANE-9-CARBOXYLIC ACID.

A. (2R*,5R*)-N-benzyloxycarbonyl-2,5-(dicarbomethoxyl) pyrrolidine (13)

10% palladium on charcoal catalyst (4 g, 10%) suspended in methanol (30 ml) was added to dimethyl (2R*,5R*)-N-benzyloxycarbonyl-2,5-dicarbomethoxyl pyrrolidine (30 g, 0.108 mol), which had been dissolved in methanol (50 ml). The resulting mixture was subjected to

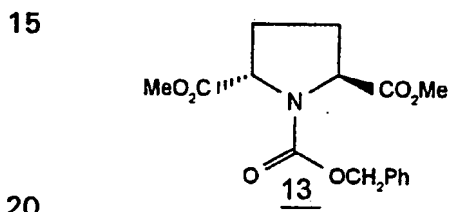
-35-

hydrogenolysis at 65 psi for 18 h. The catalyst was filtered off and the solvent removed under reduced pressure to yield (2R*,5R*) dicarbomethoxypyrrolidine (12), R_f 0.14 (1:1 EtOAc/Hexanes).



Without further purification, the product (12) (19 g, 0.101 mol), was dissolved in 100 ml of ethyl acetate. Solid sodium carbonate powder (15 g) and then benzyl chloroformate (17 ml

0.12 mol) were added to the mixture with constant stirring, which was continued for 5 h at ambient temperature. The solid was filtered off and the solvent was removed under reduced pressure to yield crude product. This was purified by flash column chromatography by eluting with hexane:ethyl acetate (3:1 mixture) to give pure (13) (26 g, 74%), R_f 0.29 (3:2 Hexanes/EtOAc).

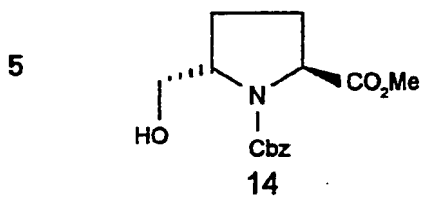


B. Methyl (2R*,5R*)-N-benzyloxycarbonyl-5-hydroxymethylpyrrolidine-2-carboxylate (14)

LiBH_4 (2.5 g, 115 mmol) was added in one lot and stirred to (13) (20 g, 62.5 mmol), which had been dissolved in 50 ml of THF and cooled to 0-5°C in an ice bath. The resultant reaction mixture was stirred at 0-10°C for 1 h and at room temperature for 3 h. The solvent was removed under reduced pressure at ambient temperature. The residue was dissolved in water (200 ml) and extracted with dichloromethane (100 ml X 3). The combined organic phase was dried over magnesium sulfate. Removal of the solvent under reduced pressure gave the crude product, which was purified by flash column chromatography by eluting with hexane:ethyl acetate mixture (3:2) to give 15g (82%) of compounds

-36-

(14), R_f 0.13 (3:2 Hexanes/EtOAc), and 1.83 g(10%) of (2R*,5R*)-N-benzyloxycarbonyl-2,5-di(hydroxymethyl)pyrrolidine, R_f 0.06 (3:2 hexanes/EtOAc).



10

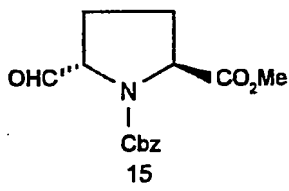
C. Methyl (2R*,5R*)-N-benzyloxycarbonyl-5-formyl-pyrrolidine-2-carboxylate (15)

Triethylamine (3.2 ml, 24 mmol) followed by sulfur trioxide pyridine complex (3.9 g, 24 mmol) were added to **(14)** (2.4 g, 8 mmol), which

15 had been dissolved in methyl sulfoxide (8 ml). The resultant reaction mixture was stirred at ambient temperature for 1 h, diluted with dichloromethane (200 ml) and washed with brine (100 ml X 2). The organic phase was dried over magnesium sulfate and the solvent removed under pressure to yield crude product, which was purified by flash

20 column chromatography using hexane:ethyl acetate (3:1) mixture to give the aldehyde **(15)** (1.15 g, 40%), R_f 0.18 (3:2 hexanes/EtOAc).

25



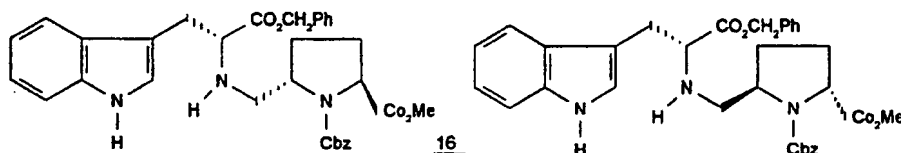
D. Benzyl N-Boc-N-[(2R*,5R*)-N'-Cbz-5-carbomethoxypyrrolidin-2-ylmethyl]-R-Tryptophan (17)

Sodium carbonate solution (25 ml, 10%) was added to R-Trp-

30 OBzl.HCl (569 mg, 1.73 mmol), which had been suspended in dichloromethane (50 ml). The heterogenous mixture was stirred at room temperature for 10 minutes. The organic phase was separated, dried over magnesium sulfate and the solvent was removed under reduced pressure

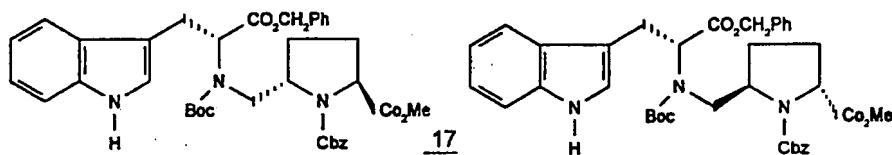
-37-

to yield the free amine. The aldehyde (50 mg, 1.73 mmol) in 5 ml of methanol:acetic acid (98:2) was added to the free amine in a 50 ml round bottomed flask. After few minutes, sodium cyanoborohydride (107 mg, 1.73 mmol) was added in two lots. The resultant solution was stirred at ambient temperature for 0.5 h and the solvent was removed under reduced pressure. The residue was taken up in dichloromethane (75 ml) and washed with sodium carbonate solution (1 X 50 ml, 10%). The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure to yield the crude product, which was purified by flash column chromatography using a hexane:ethyl acetate (3:2) mixture to give a diastereomeric mixture of benzyl N-[(2R*,5R*)-N'-Cbz-5-carbomethoxypyrrolidin-2-ylmethyl]-R-tryptophan **(16)** (700 mg, 67%), R_f 0.15 and 0.26 (3:2 hexanes/EtOAc).



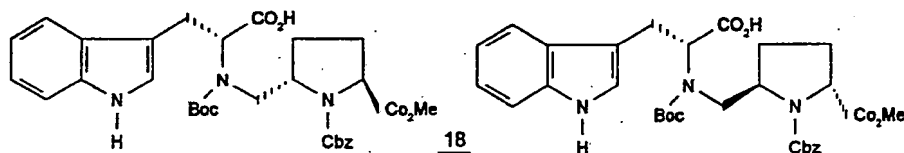
Purified **(16)** (1.1 g, 1.94 mmol) and di-tert-butyl dicarbonate (460 mg, 2.1 mmol) were dissolved in acetonitrile (4 ml) and the resultant solution stirred at ambient temperature for two days. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography by eluting with hexane:ethyl acetate (7.5:2.5 mixture) to yield the diastereomeric **(17)** (1.1 g, 87%), R_f 0.25 (3:2 hexanes/EtOAc).

-38-



E. (3R,6R,9R) and (3R,6S,9S)-2-oxo-3-(3-indolylmethyl)-4-*tert*-butoxycarbonyl-1,4-diazabicyclo [4.3.0] nonane-9-carboxylic acid (20**)**

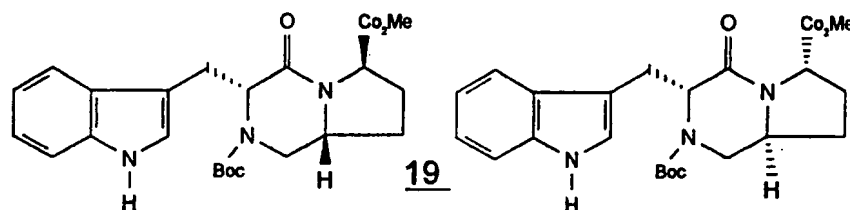
10% palladium on charcoal catalyst (200 mg, 10%) was added to a diastereomeric mixture of (**17**) (900 mg, 1.35 mmol), which had been dissolved in methanol (10 ml), and the resulting mixture was subjected to hydrogenolysis at 55 psi for 16 hrs. The catalyst was filtered off and the solvent was removed under reduced pressure to give a clear colorless oil (**18**) (500 mg, 83%).



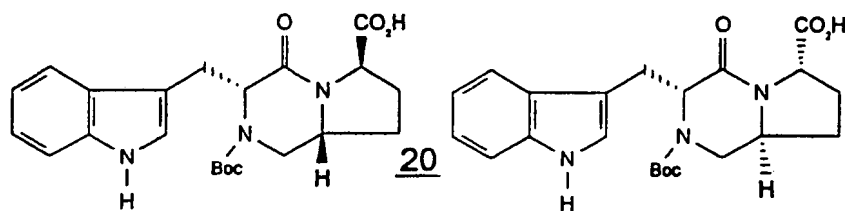
This oil (440 mg, 1 mmol), N,N-disopropylethylamine (0.2 ml) and 1-hydroxybenzotriazole hydrate (135 mg, 1 mmol) were taken up in dichloromethane (10 ml) and the mixture was cooled to 0°C. 4-Benzotriazol-1-yloxy-tris(dimethyl-amino)phosphonium hexafluorophosphate (BOP; 500 mg, 1.13 mmol) was stirred into the cooled mixture. Stirring was continued at 0°C for 1 h followed by 12 h at ambient temperature. The residue was diluted with dichloromethane (100 ml) and washed with citric acid solution (30 ml X 2, 10%) followed by sodium carbonate (30 ml X 2, 10%). The organic phase was dried over magnesium sulfate and the solvent was removed under reduced pressure. The resultant product was purified by flash column chromatography by eluting with hexane:ethyl acetate (4:6 mixture) to give 360 mg (88%)

-39-

methyl (3R,6R,9R) and (3R,6S,9S)-2-oxo-3-(3-indolylmethyl)-4-*tert*-butoxycarbonyl-1,4-diazabicyclo [4.3.0] nonane-9-carboxylate (**19**).



Lithium hydroxide solution (0.13 ml, 2 M solution) was added to
 (19) (84 mg, 0.2 mmol), which had been dissolved in methanol (0.3 ml,
 5 0.26 mmol). The resultant reaction mixture was stirred at ambient
 temperature for 2 h. Neutral impurities were extracted with ether (5 ml)
 and the aqueous layer was acidified by adding 1 N hydrochloric acid
 solution to produce a pH of about 2.5-3. The product was extracted with
 ethyl acetate (10 ml X 2). The combined organic phases were dried over
 10 magnesium sulfate and the solvent was removed under reduced pressure
 to give the compound (**20**) (74 mg, 90%).

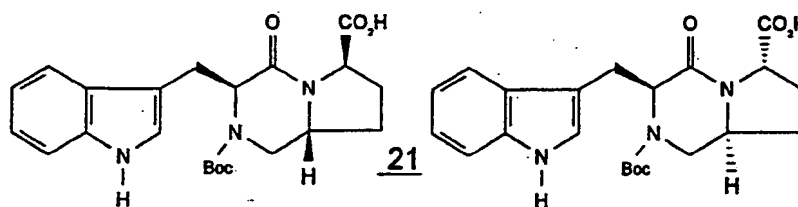


The diastereomeric mixture was separated by HPLC using a Vydax C₁₈
 column eluting with a mixture of acetonitrile and water (20-45%
 acetonitrile gradient over 30 min) to give the more mobile isomer (**20a**),
 15 retention time 14.73 min; IR: 3348, 1718, 1691 & 1641 cm⁻¹, and the
 less mobile (**20b**), retention time 17.10 min; IR: 3402, 1693, 1670 and
 1641 cm⁻¹.

-40-

EXAMPLE 3**PREPARATION OF (3R,6R,9R) AND (3S,6S,9S)-2-OXO-3-(3-INDOLYLMETHYL)-1-*TERT*-BUTOXYCARBONYL-1,4-DIAZABICYCLO[4.3.0]NONANE-9-CARBOXYLIC ACID (21).**

- 5 Following the protocol set forth in Example 2, but substituting S-Trp-OBzl-HCl for the R-isomer, compounds (21) were prepared.



The more mobile isomer (21a) has a retention time of 19.66 min (IR: 3335, 1743, 1693 and 1639 cm^{-1}) and the more polar isomer (21b) has a retention time of 22.24 min (IR: 1736, 1693, 1670 and 1629 cm^{-1}).

10

EXAMPLE 4

Compounds that are potential endothelin antagonists are screened by testing their ability to compete with ^{125}I -labeled ET-1 for binding to human ET_A receptors or ET_B receptors present on isolated cell membranes.

- 15 **A. Endothelin Binding Inhibition Binding Test #1: Inhibition of binding to ET_A receptors**

TE 671 cells (ATCC Accession No. HTB 139) were transfected with DNA encoding ET_A receptors. The resulting transfected cell lines, which express ET_A receptors on the cell surfaces, were grown to

20 confluence in T-175 flasks. Cells from multiple flasks were collected by scraping, pooled and centrifuged for 10 min at 190 X g. The cells were resuspended in phosphate buffered saline (PBS) containing 10 mM EDTA using a Tenbroeck homogenizer. The suspension was centrifuged at 4°C at 57,800 X g for 15 min, the pellet was resuspended in 5 ml of buffer A

25 (5mM HEPES buffer, pH 7.4 containing aprotinin (100 KIU/ml)) and then frozen and thawed once. 5 ml of Buffer B (5 mM HEPES Buffer, pH 7.4

-41-

containing 10 mM MnCl₂ and 0.001% deoxyribonuclease Type 1) was added, the suspension mixed by inversion and then incubated at 37°C for 30 minutes. The mixture was centrifuged at 57,800 X g as described above, the pellet washed twice with buffer A and then resuspended in

5 buffer C (30 mM HEPES buffer, pH 7.4 containing aprotinin (100 KIU/ml) to give a final protein concentration of 2 mg/ml and stored at -70°C until use.

The membrane suspension was diluted with binding buffer (30 mM HEPES buffer, pH 7.4 containing 150 mM NaCl, 5mM MgCl₂, 0.5%
10 Bacitracin, 0.1% BSA) to a concentration of 6 µg/100 µl. To this suspension 50µl of (A) endothelin-1 (for non specific binding: to give a final concentration 80 nM), (B) binding buffer (for total binding), or (C) a test compound (final concentration 1 nM to 100 µM) were added. Mixtures were shaken and incubated at 25° C for 60 minutes prior to the
15 addition of 50 µl ¹²⁵I-ET-1 (3,000 cpm). Mixtures were shaken, incubated at 4° C for 16 hours and centrifuged at 4° C for 25 min at 2,500 X g. The supernatant, containing unbound radioactivity, was decanted and the pellet counted on a Genesys multiwell gamma counter. The degree of inhibition of binding (D) was calculated according to the following
20 equation:

$$\% D = 100 - \frac{(C) - (A)}{(B) - (A)} \times 100$$

Each test was performed in triplicate.

25 Test Results

Some test results are set forth in TABLE 1:

TABLE 1

TEST COMPOUND	IC ₅₀ µM
<u>5</u>	22.5
<u>21a</u>	31.1

30

-42-

B. Endothelin Binding Inhibition Binding Test #2: Inhibition of binding to ET_B receptors

COS7 cells were transfected with DNA encoding the ET_B receptor, The resulting cells, which express the human ET_B receptor, were grown to confluence in T-150 flasks. Membrane was prepared as described above. The binding assay was performed as described above using the membrane preparation diluted with binding buffer to a concentration of 1 µg/100 µl.

The COS7 cells, described above, that had been transfected with DNA encoding the ET_B receptor and express the human ET_B receptor on their surfaces were grown to confluence in T-175 flasks. Cells from multiple flasks were collected by scraping, pooled and centrifuged for 10 min. at 190 X g. The cells were resuspended in phosphate buffered saline (PBS) containing 10 mM EDTA using a Tenbroeck homogenizer. The suspension was centrifuged at 4°C at 57,800 X g for 15 min, the pellet was resuspended in 5 ml of buffer A (5mM HEPES buffer, pH 7.4 containing aprotinin (100 KIU/ml)) and then frozen and thawed once. Five ml of Buffer B (5 mM HEPES Buffer, pH 7.4 containing 10 mM MnCl₂ and 0.001% deoxyribonuclease Type 1) was added, the suspension mixed by inversion and then incubated at 37°C for 30 minutes. The mixture was centrifuged at 57,800 X g as described above, the pellet washed twice with buffer A and then resuspended in buffer C (30 mM HEPES buffer, pH 7.4 containing aprotinin (100 KIU/ml) to give a final protein concentration of 2 mg/ml.

The binding assay was performed as described above using the membrane preparation diluted to give 1 µg/100 µl of binding buffer.

Test Results

The test compound 3-(3-indolylmethyl)-1,4-diaza-2,5-dioxo-bicyclo[4.3.0]nonane-9-carboxylic acid showed no detectable activity in the second binding inhibition assay.

-43-

C. Effect on endothelin induced contraction of the isolated rat thoracic arterial ring

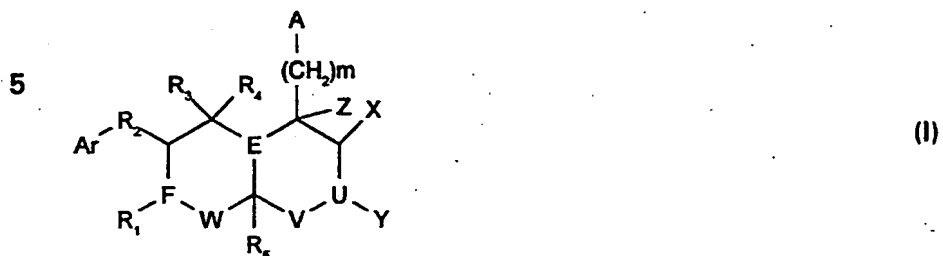
The effectiveness of the test compound as an antagonist or agonist of the biological tissue response of endothelin can assessed by measuring
5 the effect on endothelin induced contraction of isolated rat thoracic arterial ring (see, e.g., (see, Borges et al. (1989) Eur. J. Pharmacol. 165: 223-230).

Since modifications will be apparent to those of skill in this art, it is
10 intended that this invention be limited only by the scope of the appended claims.

-44-

WE CLAIM:

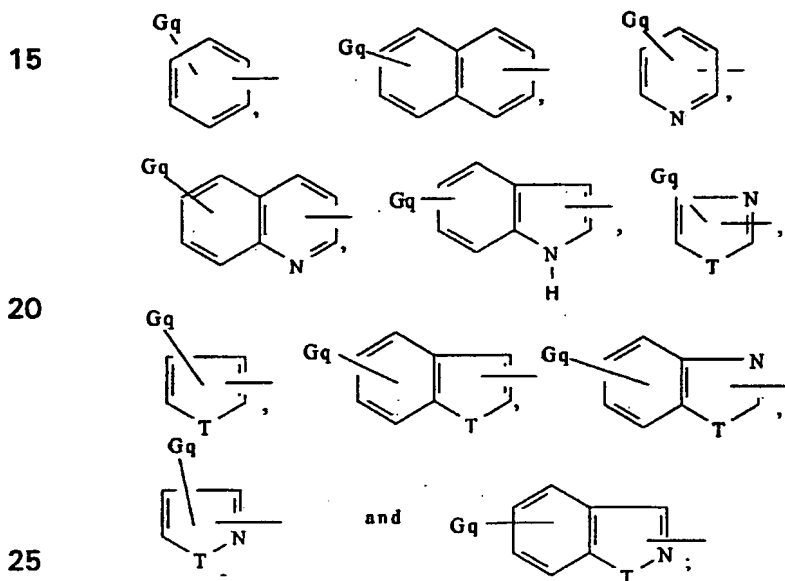
1. A compound of formula (I):



10

or pharmaceutically acceptable salts or acids of the compound of formula (I), wherein:

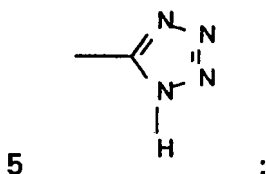
Ar is selected from the group consisting of



30

A is selected from the group consisting of COR, COOH, SO₃H, PO₃H, OH, NHR, CONHR, CON(R)₂, CONHSO₂R₁, SO₂NHCOR₁, CONHCOR₁ and tetrazole, which has the formula:

-45-



R_1 is selected from the group consisting of R, $R-C=O$, a substituted or unsubstituted aryl group, $aryl-(CH_2)_n$, halogen, R in which the

10 substituents are selected from NO_2 , PO_3H , and halogen, and R in which one or more heteroatoms are substituted in the carbon chain, wherein the heteroatoms replace one or more carbon atoms and are selected from the group consisting of O, N, and S;

R is H or is selected from the following groups that have from 1-20

15 carbon atoms: a substituted or unsubstituted straight, a branched or cyclic alkyl, alkenyl, aryl, or alkynyl group, a mixture of branched or cyclic alkyl, alkenyl, or alkynyl, an arylalkyl, an alkoxyalkyl group, and an alkoxy carbonyl group;

R_2 , which is selected independently from R_1 , is selected from the

20 group consisting of $(CH_2)_n$, CHR , $C(R)_2$, COO , OCO , $NHCO$, $CONH$, SO , SO_2 and NR ;

R_3 and R_4 , which are the same or different or each may be absent, are selected from the group consisting of $=O$, H, O-aryl, OR, O-alkyl, O-alkyl, aryl, SR, S-aryl, NHR, NH-aryl, NR;

25 R_5 is H, OH or R;

E and F, which are the same or are different, are either N or $(CH_2)_p$, and at least one of E and F is N;

G_q , which is selected independently from R_1 and R_3 , is R_1 , R_3 or halogen;

30 T is O, S, $NCOR$ or NR ;

U and V, which may be the same or different, are $(CH_2)_n$;

-46-

W is CO, $(CH_2)_n$, $(CH_2)_n-CHR$ or $CHR-(CH_2)_n$;

X and Y, which may be the same or different, are H, alkyl or aryl or
X and Y form a saturated or unsaturated homocyclic or heterocyclic ring
containing 3-15 members;

5 Z is H, SR, NHR or $N(R)_2$;

p is an integer or 0 between 0 and 5, inclusive;

q is an integer or zero between 0 and 5, inclusive;

m and n are integers or 0 between 0 and 10, inclusive;

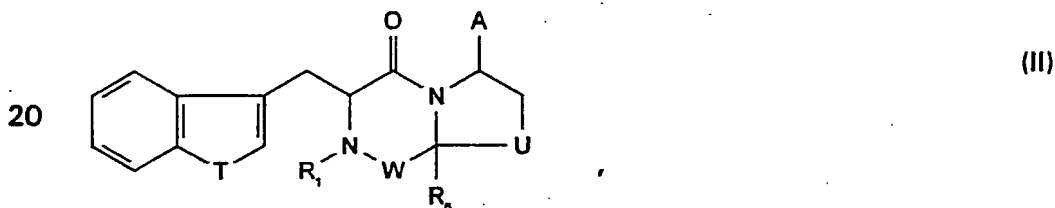
n is selected independently for each of R, R_1 , R_2 , U, V and W; and

10 n, m, p and q, may be the same or different.

2. A compound of claim 1, wherein R is a substituted or
unsubstituted straight or branched chain alkyl, alkenyl, or alkynyl group
having from 1-8 carbon atoms, inclusive.

3. A compound of claim 1, wherein R is a substituted or
15 unsubstituted straight or branched chain lower alkyl, lower alkenyl or
lower alkynyl group having from 1-6 carbon atoms.

4. A compound of claim 1 that has formula (II):



wherein:

A is COOH, CO-alkyl or CONH₂;

25 T is NH, NCH₃ or NCH₂CH₃;

W is CH₂, CH₂CHOH, or CO;

n is an integer or 0 between 0 and 4, inclusive; and

m is 0.

5. A compound of claim 4, wherein R_1 is an alkyl group.

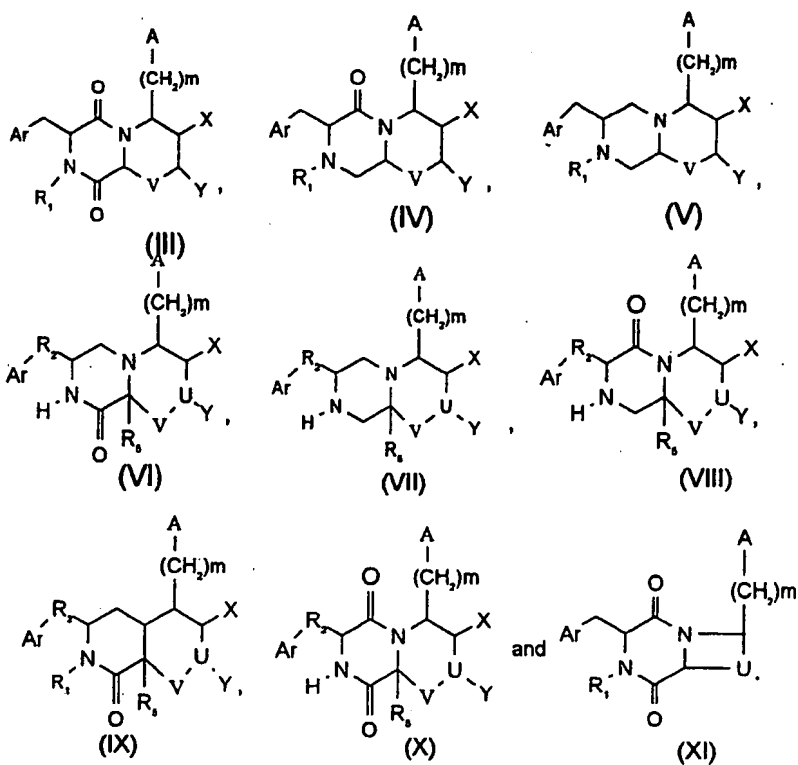
30 6. A compound of claim 4, wherein R is H.

-47-

7. A compounds of claim 5, wherein which the alkyl groups are lower alkyl groups; E and F are N; and T is NH, NCH₃ or NCH₂CH₃.

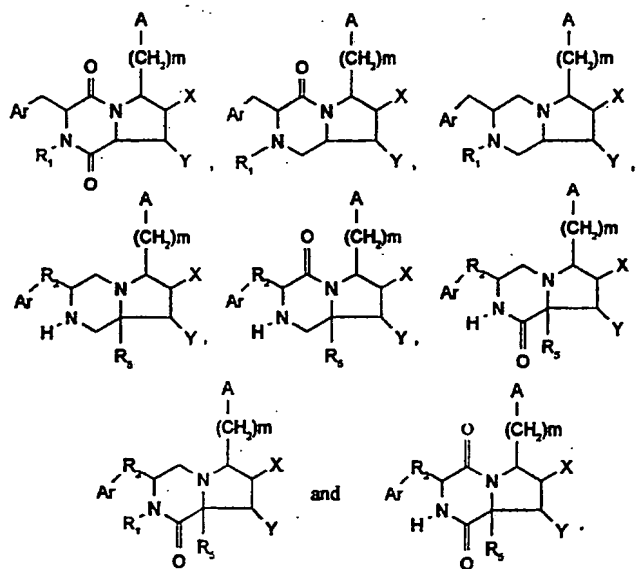
8. A compound of claim 7, wherein W is CO.

9. A compound of claim 1 selected from the group consisting of
5 any of the compounds of formulas III-XI:

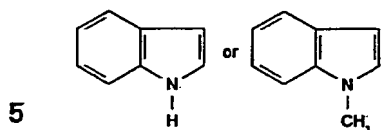


10. The compounds of claim 9 that are selected from the group consisting of

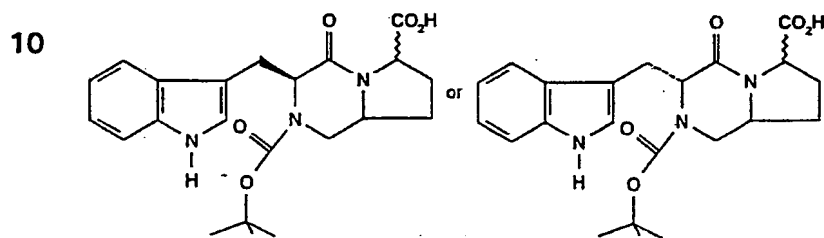
-48-



11. The compounds of claim 10 in which Ar is



12. The compounds of claim 1 that is selected from the compounds:

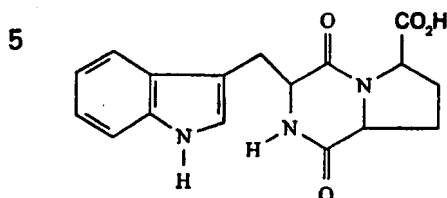


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13. A compound of claim 11 in which R_1 is 2, 2-diphenylethyl, A is COOH , m is 0, X and Y are H.

-49-

14. A compound of claim 1, which is 3-(3-indolylmethyl)-1,4-diaza-2,5-dioxo-bicyclo[4.3.0]nonane-9-carboxylic acid, which has the formula:



10 15. The compounds of claim 11 in which m is 0 or 1, A is COOH, when m is 0 or 1, or OH, when m is 0, and R, R₅, X and Y are H.

16. The compounds of any of claims 1-15 that bind to ET_A receptors with an IC₅₀ of less than about 100 μM or that bind to ET_B receptors with an IC₅₀ of less than about 100 μM.

15 17. A pharmaceutical composition, comprising a compound of any of claims 1-16 in a pharmaceutically acceptable carrier.

18. A pharmaceutical composition formulated for single dosage administration, comprising an effective amount of one or more compounds of any of claims 1-16 in a pharmaceutically acceptable
20 carrier, wherein the amount is effective for ameliorating the symptoms of hypertension, cardiovascular disease, asthma, ophthalmologic disease, gastroenteric disease, renal failure, endotoxin shock, anaphylactic shock, or hemorrhagic shock.

19. The pharmaceutical composition of claim 18, wherein the
25 compound is 3-(3-indolylmethyl)-1,4-diaza-2,5-dioxo-bicyclo[4.3.0]nonane-9-carboxylic acid.

20. A pharmaceutical composition formulated for single dosage administration, comprising an effective amount of one or more compounds of any of claims 1-16 in a pharmaceutically acceptable

-50-

carrier, wherein the amount is effective for ameliorating the treatment of endotoxin shock.

21. A pharmaceutical composition formulated for single dosage administration, comprising an effective concentration of one or more
5 compounds of any of claims 1-16 in a pharmaceutically acceptable carrier, wherein the amount is effective for reducing blood pressure.

22. A method for the treatment of hypertension, cardiovascular disease, asthma, inflammatory diseases, ophthalmologic disease, gastroenteric disease, renal failure, endotoxin shock, anaphylactic shock,
10 or hemorrhagic shock, comprising administering an effective amount of the pharmaceutical composition of any of claims 17-21 to an individual experiencing hypertension, cardiovascular disease, asthma, inflammatory diseases, ophthalmologic disease, gastroenteric disease, renal failure, endotoxin shock, anaphylactic shock, or hemorrhagic shock, wherein the
15 amount is effective for ameliorating the symptoms of hypertension, cardiovascular disease, asthma, ophthalmologic disease, gastroenteric disease, renal failure, endotoxin shock, anaphylactic shock, or hemorrhagic shock.

INTERNATIONAL SEARCH REPORT

PCT/US 93/04625

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07D487/04; A61K31/495; C07D471/04; //(C07D487/04, 241:00,209:00)		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07D ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	CHEMICAL ABSTRACTS, vol. 106, 1987, Columbus, Ohio, US; abstract no. 33114a, N. SHIMAZAKI ET AL. 'Piperazine derivatives' page 558 ; see abstract & JP,A,61 112 060 (FUJISAWA) 30 May 1986	1,17,18
A	EP,A,0 411 150 (OTSUKA) 6 February 1991 see claims 1,48	1,17,18
<p>¹⁰ Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
03 SEPTEMBER 1993		14. 09. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		ALFARO FAUS I.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/04625

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 27 is directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition"
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
On grounds of Art. 6 and 17.2a(ii) of the PCT (conciseness of claims) and of the Guidelines for Examination in the EPO, Part B; Chapter III, 2.2 (economic reasons) the search has been based on the preparation examples disclosed in the description.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

US 9304625
SA 74695

03/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0411150	06-02-91	JP-A- 3220188	27-09-91
		CN-A- 1049155	13-02-91
		WO-A- 9009380	23-08-90
		US-A- 5238938	24-08-93
		JP-A- 3173883	29-07-91
